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MINIREVIEW

Lager Yeast Comes of Age

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Alcoholic fermentations have accompanied human civilizations throughout our history. Lager yeasts have a several-century-long tradition of providing fresh beer with clean taste. The yeast strains used for lager beer fermentation have long been recognized as hybrids between two *Saccharomyces* species. We summarize the initial findings on this hybrid nature, the genomics/transcriptomics of lager yeasts, and established targets of strain improvements. Next-generation sequencing has provided fast access to yeast genomes. Its use in population genomics has uncovered many more hybridization events within *Saccharomyces* species, so that lager yeast hybrids are no longer the exception from the rule. These findings have led us to propose network evolution within *Saccharomyces* species. This "web of life" recognizes the ability of closely related species to exchange DNA and thus drain from a combined gene pool rather than be limited to a gene pool restricted by speciation. Within the domesticated lager yeasts, two groups, the Saaz and Frohberg groups, can be distinguished based on fermentation characteristics. Recent evidence suggests that these groups share an evolutionary history. We thus propose to refer to the Saaz group as *Saccharomyces carlsbergensis* and to the Frohberg group as *Saccharomyces pastorianus* based on their distinct genomes. New insight into the hybrid nature of lager yeast will provide novel directions for future strain improvement.

All human civilizations have encountered and utilized fermentation processes. Fermentations provided aroma and taste and helped to preserve food and drink. With the early stages of agriculture and the domestication of barley more than 10,000 years ago, fermented beverages were part of these societies (1, 2). Beginning with the early days of settlements, yeasts and humans shared a close association. A genetic diversity study of genotypic microsatellite loci of >650 strains suggested that *Saccharomyces cerevisiae* is a synanthropic species ("Kulturfolger" in German) that followed human settlements as a commensal in gardens and vineyards (3, 4). The process of beer making was known to the Sumerians in 6,000 BC, and in an ancient Egyptian tomb of a beer brewer in the city of Luxor, the rituals of beer making are described in more than 3,300-year-old marvelous wall paintings. Not only were fermented beverages used for religious purposes, but they were practically the only source of clean liquids, i.e., free of contamination by fecal coliform bacteria. Fermentations used for food preservation like yoghurt, sauerkraut, or sourdough bread are carried out mainly by bacteria (e.g., *Leuconostoc* and *Lactobacillus*). Alcoholic fermentation, in which the starch is converted to ethanol, however, is carried out mainly by yeasts. Among yeasts, there is quite a large variety of different species that are able to ferment sugars into ethanol, yet *Saccharomyces cerevisiae* dominates in the beer and wine industry and is also used for bioethanol production. Several types of beers can be distinguished, among them ale, wheat beers, and lager beers. Sake, ale, and wheat beer are generated by strains of *S. cerevisiae*, but lager beers are traditionally generated by lager yeasts (5–8). Ale beers have been produced since the Middle Ages. Lager beer, however, originated in the 15th century in Bavaria, became very popular in the 19th century, and today makes up the largest part of the beer volume produced worldwide. Central contributions were provided by Pasteur's discovery that yeasts are responsible for fermentation (9) and by Hansen's pure culturing of lager yeast and establishment of *Saccharomyces carlsbergensis* (10). The use of pure-culture yeasts transformed beer production into an industry.

Nowadays, studies on yeasts used in fermentation processes have increased in numbers not only due to the large economic importance of alcoholic beverages. Novel interest has been generated by genomic studies that aim at understanding the molecular details of hybridization events within different *Saccharomyces* species (11–13).

In this review, the results leading to an understanding of the hybrid nature of lager yeast and the genetic analyses that led to specific strain improvements are summarized. Lastly, the broader perspectives of network evolution on hybridizations within *Saccharomyces* species and the genomics of yeast hybrids are discussed. The extensive work on sake and wine yeast strains has been covered excellently in a recent review (14) and will not be discussed here.

IDENTIFICATION OF THE HYBRID NATURE OF LAGER YEAST

Lager yeast production strains were characterized by their low sporulation efficiency and spore viability (15). Early on, breeding of lager yeast with *S. cerevisiae* laboratory strains was viewed as an alternative (16, 17). In line with these efforts, spore-derived clones of a lager yeast production strain with opposite mating types were generated and hybridized. This started the conventional yeast breeding of lager yeasts (18). In this case, the low sporulation frequency was used to isolate "spore clones," i.e., CFU of meiotic segregants that harbored stable mating phenotypes. Later, yeast genetics of karyogamy-deficient *kar1* mutants provided a method to generate either laboratory *S. cerevisiae* strains that acquired an additional lager yeast-derived chromosome or a strain in which an *S. cerevisiae* chromosome was substituted for a lager yeast chromosome (19). Reference 19 and subsequent studies have shown (i) that a lager yeast chromosome (e.g., chromosome III

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[CHRIII]) can replace the homologous *S. cerevisiae* chromosome (e.g., ScCHRIII), (ii) that lager yeasts are heterozygous in that they harbor sequences very similar to *S. cerevisiae* and regions significantly different in sequence, and (iii) that recombination between lager yeast and *S. cerevisiae* chromosomes is strongly impaired (see reference 7 for a review). At the molecular level, sequence data for *MET2* and *MET10* provided clear evidence for an *S. cerevisiae* and a non-*cerevisiae* copy of these genes in lager yeast (20, 21, 136). This was then extended to the coexistence of two different sets of complete chromosomes in lager yeast (22). In that 1998 study by Tamai et al., genome organization of *S. pastorianus* was studied by Southern hybridization using chromosome-specific probes. This showed that in lager yeast, as in *Saccharomyces bayanus*, a reciprocal translocation between *S. cerevisiae* homologs of chromosomes II and IV can be found, thus providing evidence for the hybrid nature of lager yeast (22). Fluorescent amplified fragment length polymorphism analysis provided evidence for multiple interspecific hybridization events between *Saccharomyces sensu stricto* species (23).

By array-comparative genomic hybridization (CGH) data analysis, a persuasive candidate for the *cerevisiae* part of lager yeast genomes has been suggested to be an ale yeast (12).

Lager yeast strains can be divided into two groups, Saaz/Carlsberg and Frohberg. This division is based on the geographic heritage of the strains and was supported by molecular analyses of transposon distribution in these strains (24). Only recently, the differences in fermentation performance of these two groups were analyzed. It was shown that group I/Saaz yeasts are better adapted to low-temperature growth conditions (10°C), while group II/Frohberg yeasts ferment better at a higher temperature (22°C). Differences in sugar utilization became apparent, as group II yeasts utilize maltotriose and group I yeasts do not. Additionally, flavor differences were identified showing that Saaz strains produce severalfold-lower levels of, e.g., isoamyl acetate (banana flavor) than Frohberg strains (25, 26).

Ploidy determination of hybrid lager yeast strains has been a long-standing issue. Some advances came from a study using array-CGH and DNA sequence analysis covering several lager yeast strains (reference 12 and references therein). A 1n ploidy difference between group I and group II lager yeasts was identified. However, according to this study, group I yeasts appeared to be 2n and group II yeasts appeared to be 3n. Aneuploidy of lager yeasts and regions with copy number variations were also detected in another study using microarray hybridization (27), but only recently was it shown that, based on next-generation sequence and flow cytometry data, *Saccharomyces carlsbergensis* (group I) is essentially triploid, whereas the Weihenstephan 34/70 strain (group II) is (allo)tetraploid (26). Other studies were based on copy number variations in different strains, which also provided an estimate of the allotetraploid nature of group II lager yeasts (8).

The hybrid nature of lager yeast has been studied for decades (28). Generation of lager yeast hybrids has been attributed to man-made selection conditions of low-temperature fermentation for lager beer production. Under these conditions, a hybrid consisting of *S. cerevisiae* and a non-*cerevisiae* but cryotolerant partner may have been favored (29, 30). However, the nature of this non-*cerevisiae* species remained unclear. Based on sequence similarity, *S. bayanus* was found to be a good candidate (31). However, neither lager yeasts nor *S. bayanus* have so far been isolated from the wild. Additionally, *S. bayanus* (CBS 380^T) was found to be a hybrid between *S. cerevisiae* and *Saccharomyces uvarum* itself (32). New light was shed on this issue in 2011 by the identification of an *S. uvarum* sister species, termed *Saccharomyces eubayanus*, which bears close sequence similarity to the non-*cerevisiae* parts of lager yeasts. *S. eubayanus* was isolated from Southern beech trees in Patagonia, Argentina (33). However, insight into how *S. eubayanus* traveled to Europe and founded the rise of lager beer in 15th-century Europe is still lacking. Interestingly, *S. eubayanus* was recently also isolated near Milwaukee in Wisconsin (34). This area benefitted from English and German immigrants and generated a profound brewing industry in Milwaukee, to which lager yeast was introduced from Bavaria, Germany, by Jakob Best in 1850. However, it was suggested that the North American *S. eubayanus* strain represents a hybrid between the two Patagonian populations, whereas lager yeast strains harbor only alleles of one of these populations (34). Another candidate non-*cerevisiae* parental strain for lager yeast came from the Far East Asian corner of the world. Several strains of *S. eubayanus* have been isolated from the Tibetan Plateau, and three different lineages were identified (35). The average whole-genome sequence identity of these newly identified strains relative to sequences of the non-*cerevisiae* complement of lager yeasts was 99.82% and thus higher than that of the Patagonian strain (99.56%). Trans-Atlantic trade originated far later than trade between Europe and Asia, e.g., via the Silk Route. Thus, an Asian origin of the *S. eubayanus* lager yeast parent posts an interesting alternative to the South American variant (35).

GENOMICS AND THE ORIGIN OF LAGER YEASTS

The yeast genome sequence project opened the way for several other fungal genome projects that were boosted by new technologies and really accelerated with next-generation sequencing. This opened the way for population genomics studies for yeast (13, 36, 37). A single paper, for example, reported the genotypes of 1,000 yeast strains, including 768 offspring of meiotic segregants to generate a high-resolution meiotic recombination map (38). Undoubtedly, next-generation sequencing technology has facilitated population genetics and quantitative genetics studies and promoted comparative genomics studies (39, 40).

Compared to these large-scale approaches, lager yeast genomics has lagged far behind. One interesting finding, based on restriction mapping and sequencing, was that mitochondrial DNA of lager yeasts has been exclusively derived from the non-*cerevisiae* parent (41). With the uncertainty of the non-*cerevisiae* lager parent, molecular studies were conducted to study introgression events and the hybridization history of hybrids (42–44). *S. bayanus*, which was the best choice for the non-*cerevisiae* parent until the identification of *S. eubayanus*, was found to consist of two subgroups, termed varieties *bayanus* and *uvarum* (44). This led to the proposal to reinstate *S. uvarum* as a species within the *Saccharomyces sensu stricto* group (45). Interestingly, *S. cerevisiae* telomeric repeat sequences, termed Y elements, were found to be absent in *S. bayanus* var. *uvarum* (42). By use of gene sequencing and hybridization of *S. cerevisiae* subtelomeric sequences, it was concluded that *S. pastorianus* is more similar to *S. bayanus var. bayanus* (46). Molecular typing using random amplified polymorphic DNA-PCR (RAPD-PCR) analysis also revealed the hybrid origins of several *S. pastorianus* strains (47). These molecular studies led to the identification of *S. cerevisiae* and *S. uvarum* hybrids, particularly among wine strains (45). Finally, the *S. bayanus* type strain,

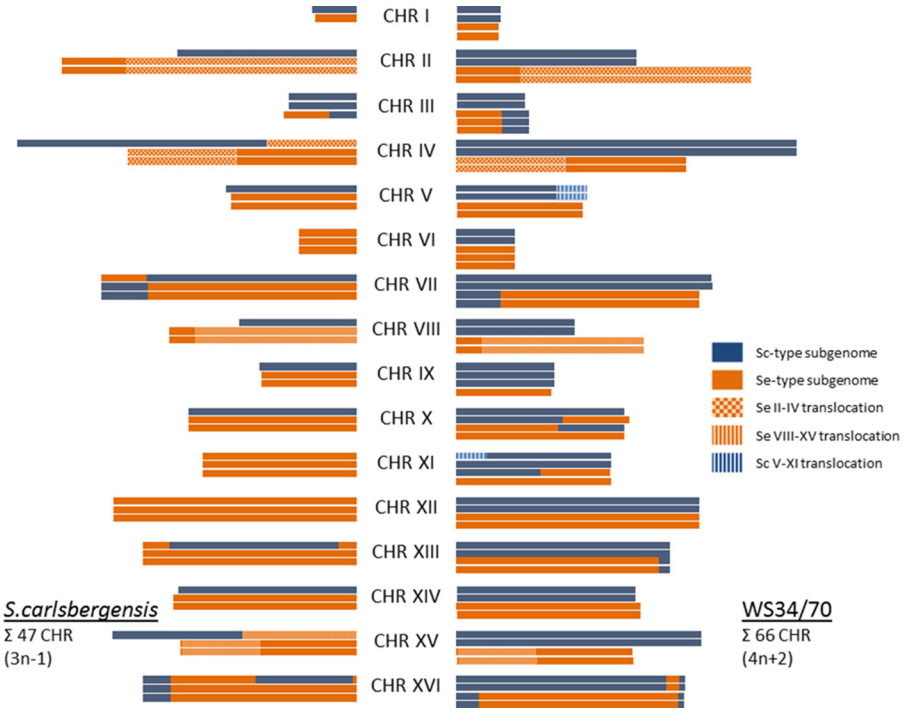

FIG 1 Map of the chromosome structures and copy numbers of *Saccharomyces carlsbergensis* (group I) and *S. pastorianus* var. *Weihenstephan* 34/70 (group II). The subgenomes of *S. cerevisiae* (blue) and *S. eubayanus* (orange) are shown. Interchromosomal translocations are highlighted. Translocations between homologous *S. cerevisiae* and *S. eubayanus* chromosomes occur at various places in both genomes. Three translocations that these strains harbor in common are on chromosomes III, VII, and XVI. *S. carlsbergensis* is basically triploid, while the *Weihenstephan* strain is essentially tetraploid.

CBS 380, was itself shown to be a hybrid between *S. uvarum*, *S. cerevisiae*, and the species now known as *S. eubayanus* (48).

Using array-CGH based on *S. cerevisiae* and *S. uvarum* CBS 7001 sequence data, Dunn and Sherlock could show the complex genome structures within group I and group II lager yeast strains (12). It became evident that the major distinctive feature of group I lager yeasts is that they exhibit a significant reduction of the parental *S. cerevisiae* genome but keep almost a full non-*cerevisiae* (i.e., *S. eubayanus*) genome. Apart from ploidy differences and copy number variations, rearrangement breakpoints were mapped. It was, furthermore, suggested that the *S. cerevisiae* parental genome was derived from an ale yeast (12). With array-CGH, chromosomal breakpoints and ploidy variations can be analyzed for gene resolution. This allowed mapping of the mosaic structure of lager yeast chromosomes (8). Copy number variations and chromosomal aneuploidies were also detected in the FostersO (with CHRIII, without CHRXIV) and FostersB (with CHRIII, -V, and -XV) ale yeasts (49).

With the draft genome sequence of Weihenstephan strain 34/70, lager yeast finally arrived in the genomics era (31). The 25-Mb genome was shown to harbor 36 chromosomes of distinct types: first, chromosomes homologous to the *cerevisiae* and non-*cerevisiae* parental genomes, second, two sets of *S. cerevisiae*-type chromosomes with translocations within a transposon yeast (TY) element (CHRV and -XI) and within conserved genes (*FRE2* and *FRE3* on CHRV and -XI), and third, eight mosaic chromosomes that were generated via one reciprocal and seven nonreciprocal translocations between the two subgenomes. These translocations indicate genome rearrangement events that occurred after hybrid formation. These data on genomic breakpoints were consistent with previous CGH data. In five cases, these nonreciprocal translocations resulted in a loss of the *S. eubayanus*-type genes. Similarly, in W34/70, the *S. eubayanus*-ribosomal DNA (rDNA) cluster has been drastically reduced in size. Furthermore, several positions located at telomeric ends were found to be involved in recombination events. These may have been mediated by TY elements, subtelomeric X elements, autonomously replicating sequence (ARS) elements, or paralogous genes (31, 50). It was shown that lager yeast can undergo genome rearrangements in response to stress, providing a persuasive argument that the translocations identified in lager yeast may have been caused by harsh fermentation conditions (51). Lager yeast genomes are dynamic in nature, and it was recently shown that group I and group II yeasts harbor different sets of translocations, indicating individual evolution histories (26). A detailed mapping of breakpoints identified the recombination positions within gene coding regions of lager yeast genomes rather than in repetitive elements or intergenic regions (52).

The genome sequence of the original lager yeast strain, *Saccharomyces carlsbergensis*, isolated by Hansen provided several key features to lager yeast genomics and evolution (10). *S. carlsbergensis* belongs to the group I lager yeasts. It is essentially triploid (3n − 1) and thus distinguishes the group I strains from the (allo)tetraploid group II strains, such as Weihenstephan 34/70. Several aspects of genome composition, e.g., the lack of *S. cerevisiae* chromosome XII, which was described previously (12, 52), were identified and, with the full genome, extended in great detail (Fig. 1). Comparison of *S. carlsbergensis* with the resequenced W34/70 genome pinpointed three translocations between the two subgenomes that are shared in both strains. This suggested that both

strains have a common evolutionary history. Group I yeasts have been associated with a regional distribution among Czech and Carlsberg breweries. Several hypotheses on the evolution of lager yeast involving multiple and independent hybridization events have been brought forward (12). While it is obvious that *Saccharomyces* species have great potential to form hybrids, a common evolutionary history of group I and group II lager strains may favor a single founding hybridization event (26). In 1845, Jacob Christian Jacobsen, the founder of Carlsberg, obtained his yeast from Gabriel Sedlmayr, the head of the Spaten Brewery in Munich, Germany. Until the year 1883, when Emil Christian Hansen selected the pure culture of *S. carlsbergensis*, these yeasts were serially repitched to produce generations of industrial fermentations. Thus, the key differences between *S. carlsbergensis* and group II strains may have evolved during these 38 years of domestication and man-made selection. *S. carlsbergensis* and the Weihenstephan 34/70 strain harbor seven and eight distinct translocations, respectively, apart from three joint translocations. It would be interesting to identify any conditions, e.g., during fermentation or storage of lager yeast strains, that could promote and select for specific rearrangements in the lager yeast genome. If there are large fitness increases associated with these common translocations found in group I and group II, they conceptually could have arisen independently. Such an adaptive evolution has been reported for nitrogen-limiting conditions (53). There are several scenarios that could explain the generation of the 3n DNA content in *S. carlsbergensis* compared to the generation of the allotetraploid group II lager yeast strains. Chromosome number evolution via chromosome (or nondisjunction) loss may result in such a loss. However, this seems unlikely to lead to the reduction of ploidy observed in *S. carlsbergensis*, as there does not seem to be a fitness advantage associated with the 3n versus the 4n state. Lager yeasts are not meiotically sterile, although sporulation is severely decreased. Historically, lager beer production was suspended over the summer months, during which sporulation may have occurred. This may have generated mating-competent diploid cells that mated with haploid cells to form such a triploid cell.

The nomenclature of lager yeast strains is not very consistent. Originally, contaminating wild yeasts in beer were classified as *Saccharomyces pastorianus* (42). DNA reassociation values showed the close relatedness between *S. carlsbergensis* and *S. pastorianus* CBS 1538, which reinstated *S. pastorianus* as a descriptor of lager yeast (54). Strain CBS 1538, however, is a group I lager yeast that was also identified by Hansen. To straighten the lager yeast nomenclature, I propose to refer to group I yeasts as *Saccharomyces carlsbergensis* and to group II yeasts as *Saccharomyces pastorianus*. This allows a clear distinction based on lager yeast genomics. With this nomenclature, Unterhefe no. 2, also known as *Saccharomyces monacensis*, would be referred to as *S. carlsbergensis* var. *monacensis*. Similarly, the *Weihenstephan* strain 34/70 would be designated *S. pastorianus* var. *Weihenstephan* 34/70. It should be noted that lager yeasts are hybrids between two defined species, but strictly speaking, by the current definition, they are not species of their own. The problems of species definition in *Saccharomyces* will be discussed below.

Next-generation sequencing will strongly promote lager yeast genomics in the future. It is expected that our knowledge of ploidy, genomic rearrangements, copy number variations, and single nucleotide polymorphisms for individual lager yeast strains will increase dramatically in the next few years. These data will promote the molecular analysis of distinct genomic changes in the fermentation performance of lager yeast strains and allow comparative studies between different lager yeasts.

TRANSCRIPT PROFILING OF LAGER YEASTS

Work on lager yeast gene expression has been lagging far behind the fast-growing number of studies utilizing genomic information on *S. cerevisiae*. The *S. cerevisiae* genome became available in 1996, whereas the first lager yeast genome sequencing effort with the Weihenstephan 34/70 strain was published in 2009 (31, 55). By that time, other large-scale sequencing efforts generated survey sequences of a large variety of *S. cerevisiae* and *Saccharomyces paradoxus* genomes (13). Lager yeast research, therefore, relied for a long time on data sets from *S. cerevisiae*.

Genome-wide expression profiling using DNA microarrays was established for *S. cerevisiae* shortly after the genome sequence became available (56–58). Although the hybrid nature of lager yeasts and the significant divergence at the DNA level between the two subgenomes was known, *S. cerevisiae*-based DNA arrays (either oligonucleotide-based microarrays or gene filtering) were used to monitor gene expression of lager yeasts under fermentation conditions (59–61). Ploidy-specific strain differences and aneuploidy-derived copy number variations are largely not reflected in the expression profiles. Furthermore, the transcriptional responses recorded for different lager yeast strains were not comparable due to differences in strain backgrounds, wort compositions, fermentation regimens, profiling methods, or fermentation stages used for the analyses (28). In general, increases in gene expression, e.g., for protein synthesis, respiration, and fatty acid synthesis, were found in the first 2 days of fermentation, which correlated with the growth phase of yeast in aerated wort. Gene expression then decreased globally as fermentations progressed, with some gene families being upregulated at the end of fermentation. Repression of stress response genes, heat shock protein-encoding genes, and alcohol dehydrogenases was found at later stages of fermentation, while upregulation of aldehyde dehydrogenase genes was noted. Interestingly, it was also found that lager yeasts apparently do not undergo changes in expression at the end of fermentation seen at the diauxic shift in *S. cerevisiae* (60, 62). On the other hand, genes counteracting oxidative stresses were found to be upregulated at this later stage, which may provide resistance to the increased ethanol content in green beer or the accumulation of medium-chain fatty acids (63, 64).

A study following gene expression during several rounds of repitching (reutilization) of the yeasts found that expression profiles were very stable, and no aging-related problems that could potentially lead to reduced fermentation performance were identified (65).

Sequencing of cDNA libraries provided the first insight into the non-*cerevisiae* part of a lager yeast genome. Sequencing identified a large number of lager genes and more than 400 lager yeast-specific genes (66). This information was then used to construct oligonucleotide arrays differentiating between *S. cerevisiae*-type and *S. eubayanus*-type genes. Of 1,000 genes characterized in this way, 400 were found to be differentially expressed across several different categories (67). A more comprehensive approach could be taken once the Weihenstephan draft genome sequence was available (31). This study clarified that the expression of most, but not all, of the *S. cerevisiae*-type and *S. eubayanus*-type homologs

correlates well throughout fermentation. *S. eubayanus*-type genes significantly overexpressed in lager yeast included genes involved in sulfur metabolism, e.g., *MET3*, sugar transport, including several *HXT* genes, and flavor production, e.g., via the production of branched-chain amino acids. Conversely, genes involved in glycolysis and ribosome biogenesis were dominated by the *S. cerevisiae* type (68).

GENETIC STRAIN IMPROVEMENT OF LAGER YEAST

Genetic improvement of lager yeast strains has aimed at increasing the performance or final beer quality under changing fermentation conditions. Genetic alterations in lager yeast are quite challenging due to the allopolyploid hybrid nature and the poor sporulation ability of these strains. Additionally, the use of genetically modified organisms (GMO) is viewed with great concern, and thus such strains are not commercialized. With recent advances in lager yeast genomics, new omics tools can be applied, and metabolic engineering and sophistication in high-throughput screening methodologies will advance the field in the future (69).

Several areas of lager yeast physiology, including sugar utilization, flocculation, reduction in off-flavor production, faster fermentation, and increase in positive flavor formation, were studied for strain improvements. Proof-of-concept studies in most cases were confined to genetically modified organisms and did not enter production (70).

The most abundant fermentable sugar in wort is maltose. Maltose utilization depends on *MAL* genes, which are clustered at telomeric *MAL* loci (71). *MAL1* paralogs encode a maltose transporter, *MAL2* a maltase that hydrolyzes maltose into its two glucose molecules, and *MAL3* a transcriptional activator of the *MAL* genes (72). Additional maltose and maltotriose transporters are encoded by the *AGT1*, *MTT1*, *MPH2*, and *MPH3* genes (73–75). Interestingly, *AGT1* encodes a low-affinity transporter that is mutated in lager yeast and nonfunctional due to a premature stop codon. Restoration of this transporter in lager yeast resulted in improved fermentation and increased alcohol production when high-gravity wort was used (76). Lager-specific Mtt1 preferably transports maltotriose over maltose and functions better at lower temperatures than Agt1 (77). This provides some explanation for the better adaptation of lager yeasts to cold fermentation conditions. Furthermore, ale *AGT1* promoters are distinguished by additional Mig1 and Mal activator binding sites in two insertion sequences compared to lager *AGT1* genes. These sites promote high-level *AGT1* expression in maltose (78).

In 1978, two groups studied the utilization of dextrin and starch by *S. cerevisiae* var. *diastaticus* (79, 80). This research identified a glucoamylase multigene family of *STA* genes (81). Improving the genetic makeup of either maltose/maltotriose transporter genes or *STA* genes in lager yeast might, therefore, enhance sugar utilization and alcohol production.

At the end of fermentation, lager yeasts exhibit a convenient feature, flocculation, that results in the settling of the yeasts at the bottom of the fermentation tank, enabling easy removal and repitching. Ale yeasts, in contrast, flocculate by rising to the surface. Flocculation is a simple process of calcium-dependent, reversible binding of flocculin proteins to mannose residues (in some cases to mannose and glucose residues) of cell walls of neighboring cells (82). Problems with flocculation arise with its untimely activation, incompleteness, or inconsistency during several rounds of fermentation. Flocculation is mediated by the activation of *FLO* genes, including *FLO1*, *FLO5*, and *FLO9*, which form one family, but also two other members, *FLO10* and *FLO11*. Flo proteins have similar three-domain structures: an N-terminal domain for carbohydrate binding, a central tandem repeat domain of varied lengths, and a C-terminal domain for cell wall attachment via a GPI anchor (83, 84). Changes in *FLO11*, namely, a deletion in the promoter region which enhances the expression of *FLO11* and an increase in the number of tandem repeat sequences in the central domain, caused these "flor yeast" cells to float and form buoyant biofilms, particularly in sherry wines (85, 86). Regulation of *FLO* gene expression is remarkably complex and generates the basis for strain-to-strain differences that can cause problems on the industrial scale (87). Several *FLO* genes, *FLO1*, *FLO5*, *FLO9*, and *FLO10*, are located at telomeres. This can influence expression of these genes via epigenetic regulation (88). Heterogeneity among *FLO* genes may occur due to telomeric recombination or via mitotic recombination involving the repeat regions, which may also include pseudogenes (89). Tuning of *FLO11* expression at the end of fermentation requires the integration of several signal transduction pathways, including protein kinase A (PKA), mitogen-activated protein kinase (MAPK), and target of rapamycin (TOR) pathways (90). One of the central transcriptional regulators is encoded by *FLO8*. Inactivation of *FLO8* in laboratory strains of S288c (due to a premature stop codon) results in lack of *FLO11* expression and flocculation (91). To ensure timely flocculation at the end of fermentation in wine yeast strains, *FLO* genes were placed under the control of stationary-phase-induced promoters derived from *ADH2* or *HSP30* (87). Taken together, flocculation is a highly evolvable trait in which specific flocculation properties can be selected via conventional yeast breeding. Nevertheless, external factors, e.g., nutrition, calcium concentration, and temperature or agitation, play important roles.

At the end of primary fermentation, most of the available sugars have been converted to ethanol. Beer processing enters the next stage, known as lagering. This is the time required for elimination of certain off flavors. Diacetyl is regarded as one of the most important off flavors in lager beers. This vicinal diketone is a byproduct of the biosynthesis pathway for branched-chain amino acids. *ILV2* plays a major role, as it generates α-acetolactate, e.g., from pyruvate. *ILV5* converts this product further. However, leakage of α-acetolactate into the medium results in its nonenzymatic decarboxylation and in the formation of diacetyl. During lagering, yeast cells reduce diacetyl into less-flavor-active substances, such as acetoin (92). A reduction of the time needed for beer maturation may, therefore, significantly shorten the overall brewing time (93). To achieve this, several routes can be taken: (i) *ILV2* can be deleted, (ii) *ILV5* can be overexpressed, or (iii) an activator of Ilv2 encoded by *ILV6* can be deleted (94–96).

Other off flavors, such as dimethyl sulfide (DMS) and hydrogen sulfide (H_2S), are derived from methionine metabolism (69). Yeasts can generate DMS from barley-derived dimethyl sulfoxide (DMSO). DMS formation can largely be avoided by deletion of the yeast *MXR1* gene (20). Reduction in H_2S and accumulation of the beneficial sulfite (SO_2) can be achieved by deletion of *MET10* alleles (97).

Yeasts produce a wide range of low-molecular-weight flavor compounds. Alcohols derived from amino acid catabolism via the Ehrlich pathway play a central role in determining the flavor of fermented beverages. This pathway contains only a few steps: con-

version of an amino acid (preferably isoleucine, leucine, valine, methionine, or phenylalanine) via transamination into an α-keto acid and then via an irreversible decarboxylation into an aldehyde, and, finally, reduction of this aldehyde to an aroma alcohol (98). In *S. cerevisiae*, multiple enzymes, e.g., members of the aldehyde dehydrogenase family, are available for each step. For the branched-chain amino acid permease gene *BAP2*, differential expression was found between the *S. cerevisiae* and *S. eubayanus* alleles (*ScBAP2* and *SeBAP2*, respectively). *ScBAP2* was highly expressed at the beginning of fermentation, while *SeBAP2* was repressed (99). Overall, an improvement in the production of natural flavors derived from, e.g., the Ehrlich pathway might be desirable in beer and wine fermentations, and screening tools to assay volatile compound formation have been developed (100). Based on consumer demands, these alterations are to be achieved in a non-GMO way using classical genetic tools and genome-based yeast breeding. Several different research roads have been pursued in this direction. Adaptive laboratory evolution, for example, has been used to generate yeast strains adapted to high osmotic stress or reduced alcohol production (51, 101, 102). Such adaptive strategies are advantageous, as mutations in several pathways can accumulate to produce the targeted phenotype (53, 103). Combination of comparative omics approaches with systems biology and metabolic engineering will provide new opportunities for improving yeast fermentations (104, 105).

YEAST HYBRIDS, POPULATION GENOMICS, AND RETICULATE EVOLUTION

After the purification of the lager yeast *S. carlsbergensis*, Emil Christian Hansen devoted many years of research to the classification of *Saccharomycetes* (106). The identification of the sexual cycle of yeasts by Winge allowed the generation of novel hybrids (107). This information was directly used to study the gene-based phenotypes, e.g., of sugar fermentations in yeast hybrids (108). The ease with which *Saccharomyces* strains can form viable hybrids results in hybrid formation also in natural environments (109–113). Based on the lack of apparent (or effective) prezygotic barriers, it has been proposed that speciation in *Saccharomyces* is ensured by postzygotic barriers preventing sporulation or the generation of viable spores. One such barrier is sequence divergence that interferes with recombination during meiosis (114–116). On the other hand, even single chromosomal translocations can contribute to reproductive isolation (117). The idea of genetic incompatibility was proposed by Bateson, Dobzhansky, and Müller (as noted in references 118 and 119). This requires two genetic loci that, when combined, result in hybrid incompatibilities (up to the point of inviability). A classic example of this is vegetative heterokaryon incompatibility in filamentous fungi (120, 121). A search for such speciation genes in yeasts revealed, e.g., an incompatibility of the *S. bayanus AEP2* gene and *S. cerevisiae* mitochondria (122, 123). However, a search for Dobzhansky-Muller pairs between *S. cerevisiae* and *S. paradoxus* did not reveal any nuclear incompatibilities (124).

Conceptually, hybrids are dead-end streets, as reproductive fitness is drastically decreased. However, even a low frequency of changes or any changes that result in an increased mating ability and the production of viable spores might initiate the route to successful speciation of such hybrids. The potential for such a breakdown of double sterility barriers and the evolution of sexually fertile lines was shown for *S. cerevisiae/S. uvarum* and *S. cerevisiae/S. paradoxus* hybrids (109, 125, 126). The fastest route to the restoration of meiotic fertility seems to be genome duplication, which enables meiotic recombination again (112).

Alternatively, hybrids may resolve by being backcrossed to parental strains, which over time may eradicate the hybrid signature and leave only traces of introgressed sequences. With genome sequences of different *Saccharomyces* species available, these introgressions can now be determined. There are already abundant examples from wine yeasts (11, 32, 49, 127). Population genomics studies have provided further evidence for the mosaic structure of *Saccharomyces* strains (13, 37). Although vineyards may not be regarded as "natural" environments, hybrid formation between *Saccharomyces* species has also been observed elsewhere, e.g., in the gut of wasp queens (113).

Interestingly, hybrids are challenged by the presence of two genomes, two proteomes, and potentially divergently evolved gene-regulatory networks, e.g., based on the divergent evolution of transcription factor binding sites (128, 129). Such gene network evolution has been addressed in a comparative study of the Ste12 and Tec1 transcription factors, which revealed a highly divergent set of target genes in *S. cerevisiae*, *Saccharomyces mikatae*, and *S. bayanus* (130).

With such data, it becomes apparent that a strict speciation model for *Saccharomyces* as defined by Mayr is inconvenient (131). In this context, members of a species were defined by their ability to produce only fertile offspring, i.e., sexually reproduce, among themselves. As seen in *Saccharomyces*, hybrid formation is a useful means of genome shuffling. This generates species complexes that can draw from a common gene pool (34, 132). The tree of life, which is based on phylogenetic relationships, thus opens into a web of life in which reticulate evolution can be displayed and also detailed through population genomics studies (Fig. 2). Furthermore, the web of life explains how hybrids can contribute to the formation of novel species and thus provide a road for diversification. There is, however, a distinction between this web of life, which is based on breeding, on the one hand and horizontal gene transfer (HGT) on the other hand. HGT can occur even between distant species. There are but a few examples of HGT in yeasts. These include, for example, the transfer of *Zygosaccharomyces bailii* sequences into wine yeast genomes and also transfer from bacterial species into yeast (133–135). Hybridization of *Saccharomyces* species followed by backcrossing to a parental strain will result in introgression of a limited amount of DNA or a limited number of genes into this parental species. The web of life provides a convenient tool to demonstrate how new species can form via hybridization and utilize a common gene pool. Interestingly, at the borders of genus-specific webs, there may be overlaps between different genera. What appears to be HGT may be an outcome of hybridization at the edges of different webs. This can be utilized for breeding in natural or in industrial settings via man-made selection. This may promote new efforts in yeast breeding that were started by Winge and Lindegren.

CONCLUDING REMARKS

Next-generation sequencing technologies have led to a rapid increase in genomic information and will also promote the analysis of the different (lager) yeast strains used in fermentation industries. Over the last years, lager yeast certainly has come of age, and future studies will enable a detailed view of natural or man-made selection and evolution of these yeast strains. Based on the web of

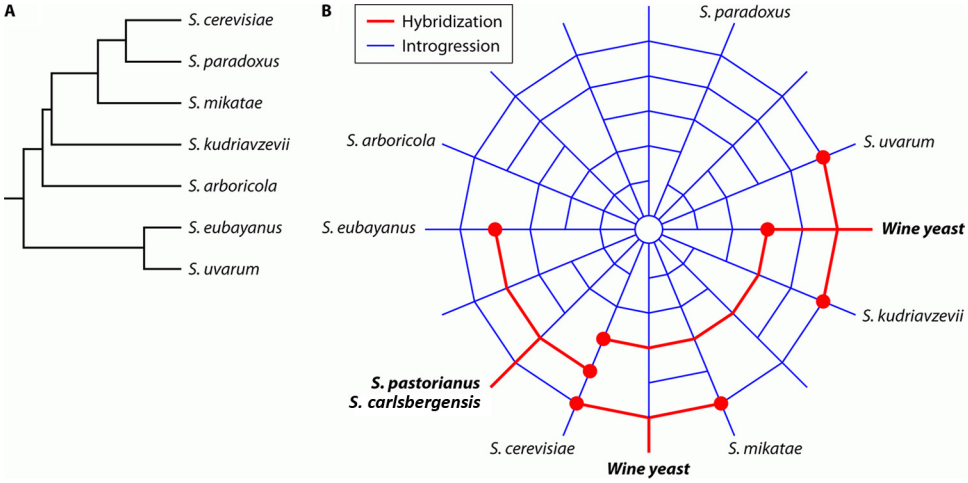

FIG 2 Phylogenetic relationships within *Saccharomyces* species *sensu stricto*. (A) Standard phylogenetic tree based, e.g., on ribosomal DNA or on more-complex multigene trees; (B) web of life of *Saccharomyces* species, which include lager yeast hybrids and wine yeasts composed of different species. Lines in red indicate parental species that contributed to hybrid formation. A background of introgression (blue) makes additional linkages and reflects the common gene pool from which hybrids can draw.

life and on the elucidation of gene network evolution, rational strain improvement strategies using genome-assisted yeast breeding can be developed.

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